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We collected at autopsy lymph nodes and tonsils from 50 drug addicts who were seropositive for HIV-1 but had no evidence of opportunistic infection or HIV-1 symptomatology. There was diffuse mild enlargement of lymph nodes, which showed B-cell hyperplasia and generalized HIV-1 RNA predominantly in a distribution of follicular dendritic cells. Quantitative polymerase chain reaction estimated viral DNA as a ratio of copies of HIV-1 to HER-2 genomic DNA. Mean HIV-1 genomic DNA ratios were 0.004 for FH-FF, 0.03 for FH+FF, 0.09 for FI and 0.08 for LS.

In surgically acquired tonsillar tissues from HIV-1 infected patients, many cells with HIV-1 gag protein were found at the surface of the nasopharyngeal tonsil or adenoid. This infected mucosal surface contained T cells and dendritic cells, which are multinucleate syncytia expressing S-100 protein and p55 dendritic cell markers.

We conclude that there is abundant HIV-1 viral DNA and RNA within solid lymphoid tissue in early stages of infection. The interaction of dendritic cells and T cells in mucosa likely drives HIVC-1 replication even in subclinical stages of infection.

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Allen P Ziegler

PI - Signature Date

1 Feb 91

INTRODUCTION

Nature of Problem. The long-term goal of this work is to establish a laboratory capable of detecting HIV-1 in tissue samples. Cellular localization as well as (semi) quantitation are parts of this goal. These techniques may be useful in staging disease and monitoring therapy. The tissues studied in this proposal will be exclusively from patients with early (i.e., non-symptomatic) HIV disease and will exclude AIDS patients.

Background of previous work. There have been several studies that have identified HIV-1 in tissues by several techniques. Immunohistochemistry and in situ hybridization have been shown to be successful in demonstrating HIV RNA in lymph nodes and central nervous system tissues. The polymerase chain reaction (PCR) has also demonstrated HIV-1 in autopsy as well as surgical tissues from patients with HIV disease. Most of these studies have been performed on tissues from patients with symptomatic HIV disease.

Purpose of present work. The initial goals of this grant project are to collect autopsy tissues from HIV-positive asymptomatic drug addicts who die unexpectedly from drug overdoses. A wide sampling of autopsy tissues was subjected to various techniques in identifying and localizing HIV-1. The viral burden, cellular sites of replication and progression of viral disease will be studied.

METHODS

Methods of approach. Three technologies will be employed: immunohistochemistry, in situ hybridization, and polymerase chain reaction. The first two techniques demonstrate viral antigens and nucleic acids, respectively, and provide information on cellular localization. PCR provides a sensitive technique for viral DNA detection and can be modified for semi-quantification of viral burden.

Case Selection. Between the months of January and April 1990, and again for the years 1991, 1992, and 1993, tissues were obtained from autopsies performed at the Maryland Medical Examiner's Office in Baltimore, Maryland with the collaboration of Dr. John Smialek. At the time of autopsy, deaths due to drug overdose were brought to our attention, and a screening ELISA for the presence of serum antibodies to HIV-1 was performed. Lymphoid, central nervous system, myocardial, and genitourinary tissues were harvested and fixed in buffered formalin and quick-frozen and stored at -80C. Tissues were harvested in both seropositive and seronegative individuals. The results of screening ELISA were confirmed at the state laboratory by Western blot methods. Thirteen consecutive seronegative drug abusers and 50 consecutive seropositive cases were subjected to complete autopsies, including gross and histologic examination of internal organs and toxicologic analysis. The largest lymph nodes from axillary, supraclavicular, mediastinal, inguinal, and mesenteric regions were measured along the long axis, bisected longitudinally and routinely processed. Cases with known acquired immune deficiency syndrome, and gross or microscopic evidence of opportunistic infection of the viscera or lymph nodes were not included. In a subset of cases, tissues from Peyer's patches (n=18), tonsil (n=28), and spleen (n=33) were also sampled.

In addition, thymus glands were studied in 12 seropositive and 9 seronegative intravenous drug abusers. An additional 5 thymus glands from seronegative trauma victims who did not abuse drugs were studied (group 3).

The gastrointestinal tracts of 26 consecutive autopsies were studied. Only individuals without a history of HIV-1 disease or seropositivity were considered for study. 17 of the autopsy cases were seronegative, and nine were seropositive. The mean age of individuals in group 1 was 37.2 +/- 2.9 years, and the mean age of individuals in group 2 was 35.0 +/- 7.1 years. 5/17 drug addicts in group 1 were female, compared to 3/9 addicts in group 2. Complete autopsies in all cases revealed no evidence of opportunistic infections, and the cause

of death in each case was classified as undetermined (narcotic intoxication).

Finally, 13 surgically acquired tonsillar tissues from HIV-1 infected homosexuals were studied for the presence of T-cells and dendritic cells, and immunohistochemistry and in situ hybridization performed on these tissues to determine the infected cell population. 11 of these patients were in the pre-clinical stages of HIV infection, with normal T-cell counts. These cases were sent in consultation to the Department of Otolaryngic Pathology, AFIP, for histopathologic interpretation.

Tissue controls Three types of positive tissues controls were used. 1. Infected H9 cell pellets acquired from SRA laboratories, Rockville, were prepared at different dilutions and frozen into cell blocks that were sectioned in a similar manner as frozen tissues or fixed and processed. 2. Human T-cell lymphoma tissues that were grown in nude mice were received courtesy of Dr. Neal Wetherall, Vanderbilt University (20); some had been infected in vivo with HIV-1 and viral replication demonstrated at Vanderbilt. 3. Frozen lymph nodes from a prostitute with HIV-1 related lymphadenopathy were obtained from the D.C. Medical Examiner's Office and positivity for HIV-1 antigens and nucleic acids established. 4. Placental tissues from seropositive and seronegative placentas were obtained courtesy of Dr. Adolfo Firpo (AIDS registry, AFIP). Negative controls consisted of seronegative drug addict tissues collected during the course of the study.

In situ hybridization. The protocol of Cecil Fox was followed with minor modifications. A full-length sheared HIV-1 RNA genomic probe was prepared by transcription and labelled with ^{35}-S labelled CTP (Lofstrand Laboratories). Both sense and antisense probes were prepared using the Promega-Biotech (Madison, WI) RNA synthesis kit. Tissue sections mounted on silanated glass slides were deparaffinized with xylene, hydrated and washed in 0.2 N HCl and digested in protease K (0.1mg/ml). Slides were acetylated with acetic anhydride and prehybridized for 2 hours at 45C in a solution prepared from 60 ml 5M NaCl, 10 ml 1.0 M Tris-HCl pH 7.4, 1 ml 0.5 M EDTA, 20 ml bovine serum albumin 5%, 2 ml Ficoll, 2 ml polyvinylpyrrolidone (PVP), 5 ml 1 M dithiothreitol (DTT), 25 ml transfer RNA (200 mg/100ml), 375 ml deionized water, and 500 ml deionized formamide. Hybridization was accomplished in a probe cocktail of 240 µl 5 M NaCl, 40 µl 1 M Tris pH 7.4, 4 µl 0.5 M EDTA, 8 µl 10% Ficoll, 8 µl 10% PVP, 100 µl 2 mg/ml yeast transfer RNA, 20 µl 1 M DTT, and 80 µl bovine serum albumin 50 mg/ml). 0.313 ml of hybridization cocktail was added to 1.25 ml 20% dextran sulfate in formamide, 0.737 ml diethyl pyrocarbonate treated distilled water, and 0.2 ml probe containing 200 million dpm. After boiling and ice quenching, probe was distributed on the tissue sections, coverslipped, and incubated overnight in a humid chamber at 45C. Slides were decoverslipped in 2XSSC (NaCl/Na Citrate, pH7.4), washed in 50% formamide and 50% 2X SSC, and then in successive washes of 2X SSC and 0.1X SSC at 60C, digested in RNAse A (40 mg/l at 37C for 40 min), dehydrated in 0.3 M ammonium acetate in 95% ethanol, and dried. Slides were exposed in the dark after dipping in Kodak NTB-2 emulsion for 14 days and counterstained in hematoxylin-eosin.

Positive control tissue sections consisted of xenotransplanted lymphoblastoid tumors grown in nude mice (obtained from Neal Wetherall, Biomed Laboratories, Minnetonka, MN) infected with HIV-1 and fixed in a similar manner to test tissues; HIV-1 infected T-cell cytologic preparations (OncorLaboratories, Rockville, MD); HIV-1 infected H9 cells mixed in a 1:100 dilution with non-infected H9 cells; fixed surgical lymph node specimens from three HIV-1 infected patients obtained for diagnostic purposes; and autopsy lymph nodes from a violent death victim with AIDS-related complex. Negative controls included slides incubated with Coxsackievirus B3 specific probes prepared with the Promega-Biotech RNA synthesis kit, slides incubated with sense HIV-1 probe, slides pre-digested with RNase, and slides pre-hybridized with unlabelled probe; all were hybridized similarly to test slides with antisense HIV-1 probe. Negative tissue controls consisted of lymph nodes collected from seronegative

drug addicts. Internal controls for the presence of RNA within tissue sections consisted of antisense RNA probes for β–actin (Lofstrand Laboratories).

Double labelling in situ hybridization and immunohistochemistry. For slides subsequently hybridized with radiolabelled RNA probes, 2X blocking serum and primary antibody were mixed in equal concentrations with RNasin (0.5 µl/20ul) and DTT (0.1M, 0.2 µl.20ul) before application to slides. The remainder of the in situ procedure was identical except that one week was used for exposure, and that hematoxylin only was used as a counterstain.

Immunohistochemistry. For immunohistochemical stains, the avidin-biotin complex method was applied to deparaffinized sections to tissues from all cases. The following antibodies (dilutions in parentheses) were purchased from Dako Corporation (Indianapolis, IN): CD35 (1:20), CD3 (1:250), L26 (1:200), OPD4 (1:50), and HIV p24 (1:25). For CD35, CD3 and p24, tissue sections were predigested with protease K at 37C (0.1 mg/ml) for 20 minutes (Sigma Chemical Co., St. Louis, MO). Biotinylated rabbit or mouse IgG was used as the secondary antibody, and the detection system was Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with Mayer's hematoxylin.

Histologic staging of lymphoid tissues. Lymphoid patterns were classified as sinus histiocytosis, paracortical expansion, follicular hyperplasia, follicular hyperplasia with fragmentation, follicular involution, and follicular depletion. Staging of lymph node changes in HIV-1 seropositive individuals followed previously published criteria. Follicular hyperplasia is characterized by numerous germinal centers with prominent mantles and tingible body macrophages. Follicular hyperplasia with fragmentation denotes the presence of enlarged crowded follicles with a depleted and irregular mantle zone; many such lymph nodes have bizarre, fused follicles with a geographic distribution. In this pattern mantles are focally disrupted, some follicular outlines are difficult to ascertain on hematoxylin-eosin stained sections, and follicles are best noted on sections stained with anti-CD35 delineating the follicular dendritic cell network. In follicular involution follicles are small, fragmented, and often scarred, measuring less than 0.8 mm in diameter; there is a lack of tingible body macrophages or mantle zones, and plasma cells are frequent within germinal centers. Follicular depletion indicates a lack of follicles and diffuse infiltration of immunoblasts and plasma cells throughout the lymph node. The degree of plasmacytosis was graded as 1 (clusters of plasma cells in the sinusoidal regions and paracortical areas), 2 (large aggregates of plasma cells filling an entire high-powered 60X field in expanded paracortical and sinusoidal areas), and 3 (sheets of plasma cells effacing the entire lymphoid tissue).

Histologic assessment of gastrointestinal tissues. In each case, representative sections of gastric body, fundus, and antrum; distal esophagus; and proximal, mid and distal rectum were fixed in 10% buffered formalin for not more than 12 hours. The number of lymphoid aggregates within the mucosa, or at the mucosal-submucosal interface was counted in each section. Those lymphoid aggregates that contained a follicular dendritic cell network, as evidenced by positive germinal center staining for CD21 and CD35, were tabulated separately. The density of lymphoid aggregates (LA) and germinal centers (GC) within the mucosa and submucosa was assessed by relating the number of each to the length of mucosa studied (cm).

Polymerase chain reaction technique. To extract DNA from paraffin blocks, a single 5-10 micron section is cut from the block and placed in a 500 µl Eppendorf tube. 400 µl of xylene is added for deparaffinization, and centrifuged for 5 minutes. The xylene is decanted, 400 µl 95% ethanol added, and decanted after recentrifugation. Acetone is added for desiccation. After drying, the tissue pellets are incubated overnight at 37C in 50 to 100 µl of extraction

solution (100 mmol/L Tris hydrochloride, 4 mmol/L ethylenediaminetetraacetate, 400 mg/L proteinase K, pH8.0). The samples are then boiled for 7 minutes to inactivate proteinase K. The samples are centrifuged, and 0.5 to 10 µl of the extraction solutions containing DNA are amplified by PCR.

To extract DNA from frozen sections, two 6-micron sections of fresh-frozen tissue are cut from the block and paced in an Eppendorf tube. A fresh microtome blade is used for every specimen to avoid carryover of DNA from one specimen to the next. The embedding medium is dissolved by vortexing the sections in 800 µl distilled water. After addition of 400 µl ethanol, the tube is vortexed again and centrifuged at 14,000 rpm for five minutes. The supernatant is decanted and the sample resuspended by vortexing in 800 µl ethanol. After another five minute centrifugation, the supernatant is decanted and any remaining ethanol is removed with a microcapillary pipet.

Cycling parameters were 1.5 minutes at 94C (denaturation), 1.5 minutes at 42C (elongation) and 3 minutes at 72C (reannealing). Reagents and kits were purchased from Perkin Elmer Cetus. To avoid contamination, positive pressure pipets were used. Tissue samples were cut with disposable blades to prevent cross-over. With each run, two negative controls were run (sterile water and negative tissue control). Positive controls included primers for human B-IFN as well as a positive tissue control. Core buffer was prepared according to kit instructions, and reaction was overlain with 4 drops mineral oil before cycling. Amplified product was detected by electrophoresis (agarose 1.5% Nu-Sieve, 3.5% Seakem), with ethidium bromide, and confirmed with P-32 labelled probes by liquid hybridization.

Quantitation of HIV-1 DNA was performed by polymerase chain reaction (PCR) using HIV-1 gag primers SK38 and 39 and performed on a frozen half of bisected lymphoid tissue. The sensitivity of the technique was determined using dilutions of T-cells from ACH-2, a chronically infected clone containing one proviral copy per cell. Quantitation was accomplished by serial 10-fold dilutions comparing amplified HIV-1 DNA product with amplified internal gene HER-2 (sense primer: 5' GGGAAAACCGCGGACGCCTG 3', antisense primer: 5' GTCCCTGTGTACGAGCCGCAC 3'). DNA extraction was accomplished by overnight Proteinase K digestion and boiling of tissue. 10-fold serial dilutions of sample DNA and serial dilutions of lysed ACH-2 cells representing 5 x 10^3, 5 x 10^2, 50, 5 and <1 cell/reaction tube were run on a Perkin Elmer cycler using standard Perkin Elmer PCR mix cycled for 30 cycles at 95°C for 30 seconds, 55° for 30 seconds, and 72° for 5 minutes. Amplified product was analyzed by liquid hybridization using ^{32}P labelled internal probes (SK19 for HIV-1, 5' GGACCTGCTGAACTGGTGTATGCAGATTGCC 3' for HER-2). Liquid hybridization was accomplished by heating 2 x 10^5 cpm of probe with 20 ul amplified product for 98°C for 5 minutes, hybridized for 15 minutes at 56°C, cooled on ice, run on 10% acrylamide gel at 20V overnight. Gels were exposed to Kodak XAR-2 film for 3 hours to overnight at -70°C. The amount of HIV-1 DNA was expressed as a ratio of maximum dilution demonstrating a positive band over internal control (HER2).

Primers and probes. Primers used for polymerase chain reaction detection of HIV-1 were the SK 29 and SK30 primers which have been shown to be sensitive and specific for HIV-1. Their respective sequences are ACT AGG GAA CCC ACT GCT and GGT CTG AGG GAT CTC TA. The probe used to identify amplified product was the SK31 sequence: ACC AGA GTC ACA CAA CAG ACG GGC ACA CAC TAC T. To determine that amplifiable DNA was extracted, an internal primer pair for human interferon is used:B-IFN GAG CCG AGC TCG CTT AAT CTC CTC AGG G (28 MER)/ GCA ACC TTT CGA AGC CTT TGC TCT GGC (27 mer).

These probes were synthesized at the AFIP by the infectious disease department, with the kind assistant of Dr. T. Hadfield.

RESULTS

Lymph node histology, seropositive cases. Eighty-nine percent of lymph nodes had a reactive pattern containing secondary follicles: 15% demonstrated FH-FF, 37% FH+FF, and 37% FI. LD was present in 9% of lymph nodes; two supraclavicular and three mesenteric lymph nodes demonstrated sinus histiocytosis. Primary follicles were rare. The distribution of the follicular pattern was similar among lymph node group, although FH+FF was more prevalent in inguinal (51%) and axillary (49%) lymph nodes compared with mediastinal (31%), supraclavicular (28%) and mesenteric (24%). The total number was similar to those of seronegative cases. However, most follicles in infected nodes were secondary follicles, whereas primary follicles predominated in control nodes. The mean size of secondary follicles per lymph node was nearly threefold greater in FH+FF than any other lymph node pattern in either seropositive or seronegative cases.

Intrafollicular and paracortical plasma cells were present in more than half of the lymph nodes and increased with progressive follicular abnormality. The increased prevalence compared to lymph nodes from seronegative controls was highly significant ($p<0.001$).

Warthin-Finckeldey type giant cells were seen in 29/50 autopsies. In 26 of these, they were present in more than one lymph node, and in 4 cases they were present in all five lymph nodes examined. Warthin-Finckeldey type giant cells were most prevalent in cases with FH+FF.

For a given individual, there was good concordance of histologic pattern among lymph nodes from diverse anatomical sites. In 14 autopsies, lymph nodes and lymphoid tissues showed FH-FF or FH+FF; the mean age of these individuals was 34.6 years. In 28 autopsies, FH+FF or FI were present; the mean age of these individuals was 37.7 years. In the remaining 6 autopsies, FI or LD were seen; the mean age of these patients was 41.3 years. In all cases histologic changes were limited to two patterns. In 17, all were of the same type (1 FH-FF, 6 FH+FF, 8 FF-FF, and 2 LD); in 10, all nodes but one were of the same type (3 LD/FI, 2 FH-FF/FH+FF, 2 FI/LD, 1 FH+FF/FI, 1 FI/FF+FF, 1 FF+FF/FF-FF, and in the remaining 23 cases there was a 2:3 distribution between two histologic patterns (15 FI/FH+FF, 6 FH-FF/FH+FF, 2 FI/LD). Dermatopathic lymphadenopathy was present 7% of lymph nodes, primarily inguinal nodes.

Lymph node size. Lymph nodes from seropositive cases were larger than those from seronegative cases ($p<0.001$, Student' t-test). Lymph nodes from seropositive cases of each histologic pattern, with the exception of lymphoid depletion and sinus histiocytosis, were also larger than those of seronegative cases ($p<0.01$). 90.2% of all lymph nodes from seropositive cases were smaller than 2.0 cm. The mean size in mm of lymph nodes from seropositive cases was 16.7 (axillary), 16.1 (inguinal), 15.0 (mediastinal), 14.9 (mesenteric), and 11.1 (supraclavicular). The mean size in mm of lymph nodes from seronegative cases was 12.1 (axillary), 12.2 (inguinal), 13.8 (mediastinal), 7.6 (mesenteric), and 6.2 (supraclavicular).

Immunohistochemistry of lymph nodes. In lymph nodes from seropositive cases, the number of CD4 positive cells decreased as the lymphoid pattern progressed. The differences from one step to the next were significant at each step ($p<0.01$, Student t-test), and the number of CD4 cells in seropositive nodes with FI or LD was less than that of seronegative nodes ($p<0.001$). The paucity of CD4 cells in FI appeared due to a decrease in interfollicular areas.

Staining with labelled anti-CD35 demonstrated the follicular dendritic cell network and follicular outlines, facilitating the classification of FH-FF, FH+FF, and FI. Staining with anti-p24 antigen demonstrated antigen in a follicular dendritic cell distribution in a minority of lymph nodes from seropositive cases. Lymph nodes showing FH-FF were most often positive (43%), followed by FH+FF (29%) and FI (19%). Staining was not observed in other lymphoid patterns or nodes from seronegative individuals.

Cytomegalovirus and Herpesvirus 1 antigen was not detected in any lymph node. Warthin-Finckeldey type giant cells were negative for endothelial markers (Qbend, Factor VIII

related antigen), histiocytic marker (Kp-1), T-cell marker (CD3), B-cell marker (L26), follicular dendritic cell marker (CD35) and interdigitating reticulum cell marker (S-100).

Histology, other lymphoid tissues, seropositive cases. Of the 28 tonsils, 7 showed FH-FF, 5 FH+FF, 10 FI, and 6 LD. Warthin-Finckeldey type giant cells were present in 12, distributed among all histologic patterns. In 11 of 18 Peyer's patches, Warthin-Finckeldey type giant cells were present. Warthin-Finckeldey-type giant cells were seen in only 2 of 35 splenic tissues. 25 spleens demonstrated secondary follicles in the white pulp; in the remainder, the white pulp consisted of small lymphocytes and plasma cells.

Histology, thymus glands. The degree of combined cortical and medullary (T-cell) thymic cellularity was similar among the three groups (seropositive drug abusers, group 1, 43.9%; seronegative drug abusers, group 2, 36.9%, seronegative controls, group 3, 38.0%). The degree of cortical atrophy was similar in all groups (mean score, 0.73, group 1; 0.80, group 2, 0.89, group 3) as was the estimate of starry sky pattern (0.9, group 1, 1.0, group 2, 1.0, group 3). There was no difference in the appearance of corticomedullary areas in seropositive and seronegative cases.

Secondary follicles with germinal centers were present only in the medullary compartments of thymus glands in the seropositive group and were present in every case (mean number 6 per section). The germinal centers were often infiltrated by plasma cells. The follicles were histologically abnormal in that they were greatly enlarged, fragmented or involuted. In seven cases, the follicular changes were compared to those of lymph nodes. In three cases, thymic follicles and lymph node follicles demonstrated hyperplasia with fragmentation; in two cases, thymic follicles and lymph node follicles demonstrated follicular involution; in one case, follicles in both the thymus and lymph nodes demonstrated follicular hyperplasia without fragmentation, and in one case there was a discrepancy between the morphology of follicles in the thymus and lymph nodes. In this case, the thymic follicles were involuted, and the majority of lymph node follicles hyperplastic with fragmentation.

The degree of plasmacytosis was most marked in the HIV-1 infected group (mean score, 1.6, group 1, 0.4, group 2, 0.4, group 3). The degree of epithelial proliferation and numbers of Hassal's corpuscles were similar in all three groups. The mean assessment of epithelial cell proliferation was 2.4 for group 1, 1.9 for group 2, and 2.4 for group 3; the mean number of Hassall's corpuscles per lobule was estimated at 5.5 for group 1, 4.7 for group 2, and 6.8 for group 3.

Quantitation of viral DNA burden. Mean HIV-1 genomic DNA ratios in autopsy lymph nodes of asymptomatic drug addicts were 0.004 for FH-FF, 0.03 for FH+FF, 0.09 for FI and 0.08 for LS.

Immunohistochemistry, thymus glands. Staining with anti-CD35 and CD21 demonstrated the extent of the follicular dendritic network within follicles. Each of the 11 seropositive cases demonstrated follicles that on adjacent hematoxylin-eosin stained sections had the appearance of secondary follicles. In only two of the seronegative thymus glands were follicles demonstrated with anti-CD35 and anti-CD21. On adjacent routinely stained sections, these follicles had the appearance of primary follicles, lacking transformed follicular center B-cells or phagocytic histiocytes. Staining with anti-p24 demonstrated intrafollicular positivity corresponding to viral localization within follicles in two seropositive cases; seronegative cases were negative. Cytokeratin demonstrated the epithelial structures of the thymus glands which were similar in all groups. Staining with anti-L26 (B cells) demonstrated follicular areas in a similar distribution as anti-CD 35 and CD21. In addition, there were scattered L26-positive lymphocytes present within medullary areas, predominantly around vessels and Hassal's corpuscles, occasionally infiltrating epithelium, in all three groups of thymuses. T-cell markers CD3 and OPD4 were diffusely present within cortical and medullary areas and did not appear qualitatively different among the three groups.

In situ hybridization, thymus glands. In ten of 11 seropositive cases, there was diffuse signal in a follicular center cell distribution within all follicles. In six of these cases, there were scattered lymphoid cells with strong overlying signal. These scattered cells, which had the morphology of mature lymphocytes, were present both within follicles as well as extrafollicularly within the medulla and cortex. There was no specific signal noted in group 2 or 3 seronegative thymus glands. These positive cells were numerically rare and ranged from 3-20 cells per section.

By in situ hybridization, HIV-1 RNA was found to be present within the lymphoid nodules and germinal centers of only 3 of 9 seropositive cases. This result is in contrast to our findings in lymph nodes in a similar population, which demonstrate HIV-1 viral RNA within follicle centers in over 90% of cases (4). The reason for this discrepancy is unclear. It is possible that the majority of lymphoid hyperplasia in the gut in asymptomatic patients infected with HIV-1 is not due to direct viral infection. Alternatively, there may be selective autolysis within the mucosa of the gut which exceeds that of lymph nodes; this autolysis may result in the degradation of RNA. In support of the latter hypothesis, qualitative histologic assessment of gut mucosa indicates that autolytic changes are significantly greater than those of lymph nodes. Because of the small numbers of individuals studied, it is further possible that a rate of 3/9 positivity wit in situ hybridization is not representative of the population as a whole. To attempt to verify one or more of these hypotheses, future studies will address expanding the total number of cases studied, as well as probing for control RNA (e.g. alpha actin) within the tissues to assess the degree of RNA degradation secondary to post-mortem autolysis.

Findings in surgically acquired tonsils. Many cells with HIV-1 gag protein were found at the surface of the nasopharyngeal tonsil by immunohistochemistry and in situ hybridization. The infected mucosal surface contained T cells and dendritic cells. The infected cells were multinucleated syncytia expressing S-100 protein and the p55 dendritic cell marker.

Gastric histology. Mucosal and submucosal lymphoid nodules were present in every group 2 stomach, and 15/17 group 1 stomachs. Germinal centers indicative of follicular gastritis were present in 11/17 group 1 stomachs and every group 2 stomach. The mean density of lymphoid nodules was 1.01 +/- 0.29 for group 1, and 3.27 +/- 0.63 for group 2 ($p = 0.002$). The mean density of germinal centers was 0.45 +/- 0.20 for group 1 and 1.2 +/-0 0.6 for group 2 ($p = 0.05$).

Rectal histology. Mucosal and submucosal lymphoid nodules were present in every rectum of either group. Germinal centers were present in 11/17 group 1 rectums and every group 2 rectum. The mean density of lymphoid nodules was 1.1 +/- 0.3 for group 1, and 2.3 +/- 0.46 for group 2 ($p = 0.05$). The mean density of germinal centers was 0.58 +/- 0.26 for group 1 and 1.0 +/- 0.27 for group 2 ($p = 0.25$).

Esophageal histology. Mucosal and submucosal lymphoid nodules were present in all esophagi from group 2 and 15/17 esophagi from group 1. Germinal centers were present in 9/17 group 1 esophagi and every group 2 esophagus. The mean density of lymphoid nodules was 1.1 +/- 0.4 for group 1, and 2.2 +/- 0.6 for group 2 ($p = 0.16$). The mean density of germinal centers was 0.76 +/- 0.34 for group 1 and 1.25 +/- 0.55 for group 2 ($p = 0.44$).

In situ hybridization, gastrointestinal tissues No signal was noted in any seronegative case (17/17) in any of the three organs studied. In three of the nine seropositive cases, positive signal, consisting of marked increased density of silver grains, was noted over lymphoid aggregates and germinal centers. The distribution was similar to that previously reported in lymph nodes (1). In two of the three cases, signal was present in esophagus, stomach, and rectum; in one case, signal was present in stomach and rectum only.

CONCLUSIONS

The aim of this study was to determine the extent of histopathologic changes and presence of HIV-1 viral RNA throughout the lymphoid system in early HIV-1 disease. We found that a high percentage of lymphoid tissues demonstrate follicular hyperplasia with fragmentation and follicular involution characteristic of HIV-1 infection. Follicular fragmentation, involution or depletion are generally present throughout the lymphoid system and there is good concordance from one lymphoid site to another. Therefore, sampling of one lymph node group may be indicative of overall histopathologic pattern. Our data do not indicate that early HIV-1 related histopathologic changes are concentrated in certain lymphoid sites but suggest that HIV-1 infection is multicentric from an early stage of infection.

The histopathologic changes of HIV-1 infection are not specific, but certain features are suggestive of HIV-1 disease and were not present in our control samples. Warthin Finckeldey syncytial cells, which are present in hyperplastic or atrophic nodes, were absent in non-infected tissues. Hyperplastic follicles with a marked degree of fragmentation, loss of mantles, and irregularity with a geographic pattern, were also absent in control tissues. Lymphoid tissues with involuted or depleted follicles are similar in histologic appearance to quiescent nodes from seronegative controls, but generally demonstrate a much higher degree of plasmacytosis in medullary areas. Although we have demonstrated that lymphoid changes are fairly distinct from a group of non-infected individuals without evidence of lymphoid disease, we did not compare HIV-1 related histopathologic changes to other infectious processes involving the lymphoid system. The specificity of HIV-1 induced changes can only be discussed in the context of a particular patient population.

The clinical stages of HIV-1 disease includes a very early transitory stage of lymphadenopathy (AIDS-related complex). Because none of our cases had palpable lymphadenopathy, and because seropositive drug addicts would be expected to overdose at all stages of early HIV-1 disease, it is likely that the stage of AIDS-related complex is of short duration in comparison to the clinically latent stage before significant immunodeficiency. With the exception of mediastinal nodes, early stage lymph nodes in our study were slightly but significantly larger than seronegative controls. These data indicate that there is a prolonged stage of mild adenopathy in early HIV-1 disease. The histopathologic patterns of follicular hyperplasia and fragmentation, as have been described in patients with clinically enlarged nodes and AIDS-related complex, are found in this phase of mild adenopathy as well.

There are few data regarding in situ hybridization results in non-enlarged lymph nodes from HIV-1 infected individuals, or from several lymphoid groups from the same HIV-1 infected individual. Previous reports of in situ hybridization have predominantly utilized surgically acquired single lymph nodes from a highly selected population with lymphadenopathy. In early infection, we have found HIV-1 RNA by in situ hybridization in diverse lymphoid tissues when follicles containing follicular dendritic cells were present. Certain lymphoid sites, such as tonsils and inguinal nodes, were likely to contain HIV-1 RNA, because of a high prevalence of reactive or involuting follicles in these tissues. HIV-1 RNA was also present in splenic tissues demonstrating follicular hyperplasia, a finding not previously reported. The three stages of HIV-1 related lymphoid disease with CD-35 positive germinal centers, namely follicular hyperplasia, follicular hyperplasia with fragmentation, and follicular involution, had HIV-1 RNA within germinal centers in a follicular center cell distribution. This distribution was demonstrated to correspond to the follicular dendritic cell network by comparison with serial sections stained with anti-CD35. The signal is typically extracellular corresponding to follicular dendritic cell processes.

In our tissues, the strength of this signal decreased with the later patterns of lymph node changes, namely follicular fragmentation and lysis. Our data suggest that there may be a progressive recruitment of lymph nodes infected with HIV-1 until the stage of follicular involution, in which HIV-1 RNA is decreased due to gradual destruction of the follicular dendritic network. Similar to other observers, we did not note positive signal in nodes with follicular depletion.

In addition to diffuse follicular dendritic cell staining, we also demonstrated signal over lymphocytes within germinal centers and interfollicular areas. Scattered intra- and interfollicular cells demonstrating signal for HIV-1 RNA have been demonstrated in the absence of follicular dendritic cell staining, have been shown to label more strongly than follicular dendritic cells, and have been seen in all histologic patterns of HIV-1 infection . In double labelling with immunohistochemistry, these cells have been found to represent CD4 positive T-cells. If these cells truly represent circulating helper T-cells that express HIV-1, they appear to be numerous only in early stages of follicular hyperplasia and are depleted at the stages of follicular involution and depletion. This finding may have relevance in staging of lymphoid tissues in HIV-1 disease and needs to to correlated with the level of expression of HIV-1 RNA in circulating T-cells, which is believed to be very low, on the order of 1:1000 cells.

Our gastrointestinal studies show that there is also diffuse follicular hyperplasia in the esophagus, stomach, and rectums of drug addicts who are seropositive and who show no signs of AIDS. Compared to non-infected controls, the density of lymphoid aggregates and germinal centers within the mucosa of HIV-1 infected drug addicts was greater for all three organs.

Our findings in surgically acquired tonsils demonstrated the presence of infected mucosal dendritic cells, indicating that these may contribute to transmission. We are uncertain why these were not present in the autopsy acquired tissues, but may be related to the fact that the surgical cases were primarily from homosexuals.

Asymptomatic infection with HIV-1 is characterized by a generalized mild lymphadenopathy and hyperplasia of the B-cell areas of lymph nodes, mucosa-associated lymphoid tissue, and the thymus. Viral RNA burden is concentrated in lymphoid follicles in these tissues, and is present in mucosal dendritic cells, which may represent a route of infection. Because of the diffuse nature of lymphoid proliferation as well as viral RNA accumulation within these tissues, the viral burden in early HIV-1 disease appears to be staggeringly high.

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